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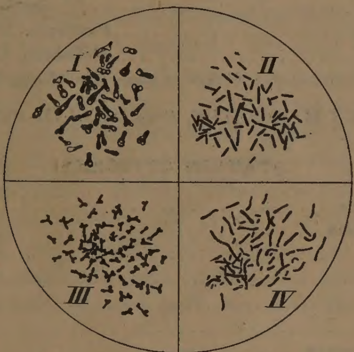

FOUR FORMS OF NITROGEN-GATHERING BACTERIA

Soil Inoculation With Artificial Cultures

BY

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Soil Inoculation with Artificial Cultures.

INTRODUCTION.

The greater part of the soil of Virginia, which once bore a magnificent forest and later produced wonderful fields of tobacco and corn, is becoming very much depleted. The long years since 1607 of continuous cropping and neglect have, in some sections, almost entirely exhausted the elements of plant growth once so bountiful and these must be restored before crops, or even forests, will again flourish. Among the exhausted elements, nitrogen is the most expensive and difficult to recover. The principal means for restoring nitrogen to depleted soils are, (1) application of barnyard manure, (2) use of commercial fertilizers, and (3) growing of leguminous crops which have been infected with nitrogen-fixing bacteria and which take nitrogen from the atmosphere and store it within the plant.

The supply of barnyard manure is insufficient to make up for the losses of nitrogen occasioned by ordinary cropping. The world's supply of the two richest sources of nitrogen, saltpetre and guano, is being rapidly exhausted. Guano has already ceased to be of any great importance; and while it is difficult to obtain a precise estimate of the amount of available saltpetre, it is believed that at the present rate of its consumption it cannot last for a very great length of time, the most optimistical placing the limit between twenty-five and fifty years. It should also be remembered that the natural product, while so rich in nitrogen, is also so expensive that for the general farmer the cost is almost prohibitive. The same may be said of the process recently proposed for the manufacture of nitrogen salts by means of electricity. While the discovery and perfection of such a method is calculated to calm the fears of those who predict a nitrogen famine, it is not one that appeals to the farmer, so long as the price is not reduced. The

small amount of ammonia, nitrous and nitric acid, and of nitrogen fixed by lightning in a form suitable for plant food is too insignificant to be considered. Finally, this brings us to the growing of legumes with bacteria for fixing the nitrogen of the atmosphere, as a feasible solution of this serious question.

Former experiments showed that leguminous plants obtained nitrogen from some source and under conditions where it was not available for the nutrition of the cereals, and, eventually, that it was obtained from the atmosphere. It was suggested that the tubercles observed on the roots of leguminous plants had a direct relation to the appropriation of nitrogen, but most observers looked upon them as abnormal and of no physiological significance. The latest investigations, however, show beyond the shadow of a doubt that these tubercles or nodules are the result of infection by microbes and that the relation between the roots and the bacterial organisms is a true symbiotic one, each developing more vigorously at the expense of the other, and that free nitrogen is appropriated by the microbes.

THE BEGINNING OF SOIL INOCULATION.

In 1883 Hellriegel began experiments with leguminous plants in pots of washed quartz sand to which no nitrogen was added. Marked differences were observed in the growth of the plants under these conditions, and tubercles were found on the roots of the plants that made the best growth, while they were absent in other cases. He was then led to attempt the production of the root tubercles by seeding or inoculating sterilized sand with a water extract of a soil in which leguminous plants were growing. To some of the pots in which peas and vetches were planted from 25 to 50 c. c. of a water extract of a fertile soil was added. When this soil extract was not sterilized there was a luxuriant growth of the plants in the pots to which it was applied, with abundant formation of the root nodules; but when the soil extract was sterilized, this result was not obtained. This soil extract, however, was without effect on lupines and some other plants; but when the lupine pots were inoculated with an extract of a soil in which lupines were growing, the plants

No. I. Alfalfa plants; those on the right not inoculated; those on the left inoculated and fertilized with 16 per cent. basic slag at the rate of 500 pounds per acre. The uninoculated plants have no nodules.

made a luxuriant growth and the root tubercles were abundantly developed. In all cases the nitrogen supply of the plants was coincident with the development of the root tubercles that were produced by inoculation with an extract of a fertile soil.

Thus we see that for more than twenty years it has been known that legumes possessing nodules on their roots have the power to take nitrogen from the atmosphere and make use of it in the actual growth of the plant. Plants possessing these nodules, or a bacterial infection without the formation of nodules, were found to be the only ones capable of this action. The organism or germ present within the nodule, and the air, were found responsible for the formation of such enlargements and also for the collecting and transforming of nitrogen into an available form.

From the fact that these nodules or tubercles will form only in soils infected with the nodule-producing organism, it is impossible to grow legumes possessing such nodules in soils where that particular legume, or one closely related to it, has not previously been grown, and the proper organism distributed. However, a complete inoculation is made possible by persistent cropping of one field to the same legume. The nodule-forming bacteria are carried in small numbers on the seeds from their respective hosts; consequently, by the application of one kind of seed to the same field year after year, a number of germs are distributed, which, together with those that have developed on the hosts of previous seasons' planting, finally succeed in effecting a uniform inoculation over the whole field. This method of securing a successful inoculation, although a rather long and expensive one, has proven very successful in a number of cases which have come under our observation.

Where the land does not contain the proper organisms in sufficient quantity, it becomes necessary to supply them by means of inoculation. Soil taken from a field on which nodule-laden legumes have grown for some years with success, was the first means used for inoculating another field with the proper organisms. This method usually proves very satisfactory from the standpoint of securing proper inoculation, but it has its objectionable features. The expense incurred in shipping and applying sufficient quantities of the germ-laden soil to secure thorough inoculation is often high,

and the true germ content of such soil is often questionable. These objections, however, are counteracted to quite an extent by the effectiveness of the method.

ARTIFICIAL CULTURES.

Several years ago Nobbe, a German investigator, conceived the idea that inoculation might be brought about by pure cultures grown in an artificial medium. He put his idea into practice and sent out cultures of the organism isolated for each kind of legume. These cultures were distributed in bottles on a neutral medium. The use of these cultures in Germany gave many favorable results; a new field had been opened, much broader, perhaps, than scientific men at that time realized. Tests of his cultures were also made in the United States, with a less degree of success than had been met with in Germany, but it failed to receive general support because of the questionable results. Caron, of Ellenbach, at the same time prepared an inoculating material known as *alinit,* which was widely distributed and met with varying degrees of success.

The German investigators continued to improve the methods of inoculating with artificial cultures. Hiltner, in June, 1903, read a very interesting article on "The Practical Importance of Inoculating Legumes with Pure Cultures,"* in which he reports for the year 1901, from forty-five field experiments, that 54 per cent. were successful; for 1902 as follows: Serredella, 78 per cent. successful; red clover, 75 per cent.; lupines, 57 per cent. Of one hundred and eighty other field experiments he secured an average of 60 per cent. successful inoculations. He declared upon this occasion that successful inoculation depended upon the quality of the cultures, that is their virulent strength. It was found that cultures made from plants which had grown for three or four generations in soil somewhat deficient in nitrogen were much more vigorous and gave better results in practice than others which had been prepared from such hosts. Fischer, in 1902, recommended a nitrogen-free solution for growing virulent, spore-building, nitrogen-fixing bacteria.

With these facts before us, the writer, in 1903, for the Virginia Experiment Station, began preparing in the laboratory inoculating

*Berichte Angewandte Chemie 1903, No. III.

material from pure cultures for several of the most common legumes and conducting experiments to test the practical value of same. Our experiments have confirmed the statement, previously made, that nitrogenous media, such as are used in the laboratory, have much to do with lessening the vigor of nitrogen-fixing bacteria, both in their power to produce infection and in storing up nitrogen. Consequently we have used only media which were free from nitrogen in the production of our cultures. We have also found by our experiments that not only do the nitrogen-fixing bacteria produce more vigorous cultures in nitrogen-free media, but that a slight difference in the formulæ of the nitrogen-free media makes a very material difference in the growth and development of the bacteria associated with the different legumes. In order to produce the best cultures for alfalfa, the clovers, peas, and vetches, we found it necessary to employ at least four different formulæ, which we give below under the numbers used in the laboratory.

MEDIUM NO. 120.

Tap water	1.	litre
Biphosphate of Potash	1.	gram
Magnesium Sulphate	.1	gram
Cane Sugar	10.	gram
Common Salt		trace
Iron Sulphate		trace
Manganese Sulphate		trace

MEDIUM NO. 130.

Tap water	1.	litre
Biphosphate of Potash	1.	gram
Magnesium Sulphate	.1	gram
Glucose	20	grams
Common Salt		trace
Iron Sulphate		trace
Manganese Sulphate		trace

MEDIUM NO. 140.

Tap water	1.	litre
Mannite	20.	grams
Biphosphate of Potash	1.	gram
Magnesium Sulphate	.1	gram
Common Salt		trace

No. II. Alfalfa plants; those on the right not inoculated; those on the left inoculated and limed at the rate of 1,000 pounds per acre. They also show the nodule formation. The uninoculated plants have no nodules.

MEDIUM NO. 150.

Tap water..	1.	litre
Cane Sugar	10.	grams
Biphosphate of Potash	1.	gram
Magnesium Sulphate	.2	gram
Common salt	.5	gram
Iron Sulphate		trace
Manganese Sulphate		trace
Calcium Chloride		trace

These media were placed in 1-litre flasks and subjected to intermittent sterilization; that is, sterilizing for twenty minutes each day on successive days in the ordinary sterilizer. The solution being sterile, it was then easy to grow a culture free from contamination.

The method we pursued was as follows:

Fresh nodules are secured from host plants grown in coal ashes or sand containing no nitrogen, thus giving a high degree of virulence with which to start. These are freed from soil, then with distilled water and brush, repeatedly washed, after which they are placed for a few minutes in a solution of 1 to 1000 mercuric chloride, rinsed in sterile water and dried between folds of filtered paper. This being done, the nodule is taken between sterile forceps, merged for an instant in alcohol, allowed to dry, and cut open with a sterile knife. Then from the center of the nodule the medium is inoculated with a needle in the ordinary manner. In case of very small nodules, they are washed and sterilized, and then mashed between points of forceps and transferred to the medium. The cultures are then placed in an incubator ranging from 25° to 30°C. Within 24 to 36 hours the liquid media usually become cloudy in appearance. Then a microscopic examination is made and the bacteria found are compared with those found in the nodules of the legume from which the culture has been made. We find that by following this method pure cultures may be obtained directly from nodules, and very seldom have we been obliged to discard them on account of contamination. After 72 hours the culture is ready to inoculate seed or for preparing the material for distribution. The practical tests made with these cultures on the Station grounds have given very gratifying results, as illustrations Nos. 1 and 2 and 3 will show.

When the commercial cultures of nitrogen-fixing bacteria suddenly came on the market in the spring of 1905, we were in position to prepare cultures which we felt sure would be equal if not superior to any others made; and in order to check the wild enthusiasm caused by the very extensive and misleading advertisements, we hastily prepared and offered to the farmers of Virginia the inoculating material at a sum sufficient to cover the cost of preparing, packing, and mailing, the packages being put up in three sizes, for one, two, and three acres respectively, with directions for use.

In this way, during the spring and summer of 1905, material for inoculating 3,737 acres was distributed over different portions of the state, at a cost to the farmers of $934.25, instead of $7,474.00, the amount the same quantity of nitro-culture would have cost if purchased from commercial concerns, thereby saving to those who used it the sum of $6,539.75.

The manner in which the cultures were prepared in spring of 1905 seems to be fully endorsed by the U. S. Department of Agriculture, as that Department is sending out, this year, liquid cultures in sealed tubes with a time limit of life, which is practically the same method employed by the author one year ago. However, the dry cultures have given a fair degree of satisfaction and, because of the convenience in shipping, a number of these will also be made for the coming season.

RESULTS OBTAINED FROM THE INOCULATING MATERIAL.

All our press bulletins and letters regarding the distribution of the inoculating material made particular mention of the fact that we desired everyone who used it to make a careful test of its efficiency; for, after all, the value of any such product must be determined by its efficiency under various conditions when used on the farm. In November, 1905, the following circular letter with questions attached was sent to all farmers who used this material:

VIRGINIA AGRICULTURAL EXPERIMENT STATION.

Dear Sir: Blacksburg, Va., Nov. 1, 1905.

The Experiment Station desires to obtain all information possible concerning the value of inoculating the seeds of le-

gumes with nitrogen-fixing bacteria. This is of material interest to every farmer in the state, and as you are one of the many who used the inoculating material sent out from the Station this year, we earnestly request you to give us the results of your experiment by answering the questions on this sheet and return to us at your earliest convenience in the stamped envelope enclosed herewith.

Crop................ Amount seed per acre...............
Date of planting........... Date of harvesting ....
Preparation of seed bed.......................................
Previous crop grown on land....................................
Type of soil...
Did you subsoil?............What fertilizer used, and how much per acre?................Did the seed come up well? ....Were nodules formed on the roots?........Date when examined........ Did you harvest a good crop?...... Do you think your crop was benefited by the inoculation?.... ....Do you consider that the inoculating material had a fair trial? ....Did you compare inoculated with uninoculated plats?................Please state if the crop was injured by wet or dry weather, weeds, or from any other cause...
Remarks...

Three hundred and forty-four have been returned with questions answered. Of these, we have 234 which indicate that the inoculating material was, without doubt, a success, and that the crop was benefited by it. Twenty-seven showed that the soil already contained the proper bacteria and that the crop was not materially benefited by the inoculation. Twenty-two did not show any effect of the inoculation, neither by formation of nodules or increase in crop. Sixty-one stated that there was no crop, due to bad seed, bad season, or weeds and grass. From the above we have the folling percentages:

Benefit derived from inoculation	82%
No benefit from the inoculation	8%
Land already inoculated	10%

The 61 crop failures due to the conditions stated are not contained in this table.

No. III. Two plants of snap beans grown in coal ashes ; the one on the right inoculated ; the one on the left not inoculated. Notice large mass of nodules on the inoculated plant.

This is a very good showing, and is somewhat better than that claimed by the Department of Agriculture at Washington; also averages better than the experiments conducted by the different stations of Germany in the year 1903. With these results, we feel that our efforts to secure an inoculating material which would meet the conditions existing on the farm have been successful. We believe that this has been due to the manner in which we proceeded in securing vigorous cultures. This procedure, you will notice, is as follows: We have grown our host plants in coal ashes, or sand, supplying them with no nitrogenous elements, always looking to the bacteria present to supply this. This has given us nodules produced by very strong, vigorous bacteria, which in turn have been grown in a liquid, nitrogen-free medium and again forced to gather nitrogen from the air for their sustenance. Thus we have cultures which have for two periods in their development, one on the plant and the other in the artificial medium, depended on their own resources for nitrogen.

WHEN INOCULATION MAY BE BENEFICIAL.

Since it is no longer questionable that the seeds of legumes can be inoculated with artificial cultures of nitrogen-fixing bacteria and large amounts of nitrogen gathered from the air and converted into forms, not only available for the growing crop but stored in the land for the use of future crops, it would be well for us to note the conditions under which the best results may be obtained.

1. On poor land that has not previously grown legumes.

2. On land that produces poor crops of legumes and where, upon examination, the roots fail to show the presence of nodules.

3. Where the legume to be planted is of a widely different species to the ones previously planted on the land.

4. Where the land has previously produced a lot of legumes, possessing nodules which, instead of being beneficial, acted as parasites. Good results may be obtained from the use of pure cultures when a field which has previously grown good crops of legumes begins to give evidence that, all other conditions being the same,

it is not producing the highest yields. The cause may be that the bacteria already in the soil are losing their virulence, and the only way to be certain of this is to try inoculation and note results.

When Inoculation is Unnecessary.—Since the only purpose in adding the bacteria to the soil is to furnish nitrogen to the plants in an available form, usually with root nodules, it is evident that where the organisms are already abundant and the crop is thriving, but little benefit can be expected from an additional inoculation. Neither is inoculation necessary where the soil is already very rich in nitrogen, nor where it is already full of nitrogen-fixing bacteria.

WHEN THE INOCULATION WILL BE A FAILURE.

1. When the directions for preparing the solution are not carefully followed.

2. When the soil is too acid or too alkaline to permit the development of either plants or bacteria.

3. When the soil is deficient in other necessary plant foods, such as potash and phosphorus, as well as nitrogen.

Above all things, it should be remembered that no inoculation will overcome poor results due to bad seed, improperly prepared seed beds, bad cultivation of land, weeds and grass, or adverse climatic conditions. Liming where needed and the addition of phosphatic and potash fertilizers should not be neglected.

We have a large amount of data to show that where inoculation is successful it is of great value to farmers. As concrete examples, Dr. Hopkins of Illinois reported that by inoculation 90 lbs. of nitrogen per acre was added to the soil; at the market price, the nitrogen gathered in this manner was worth $13.50 per acre. A German investigator reports that on two occasions the nitrogen stored in the soil by means of inoculation was worth $18.00 per acre.

*Mr. J. F. Strauss, of Washington, D. C., made the drawing for the frontispiece from slides prepared by the author.

SUMMARY.

1. Inoculation can be done successfully and profitably where care is taken in observing the proper methods.

2. A conservative course is recommended. The recent wild enthusiasm, using such terms as "vest pocket fertilizer," "act like magic," etc., is misleading; while any statement that inoculation by artificial cultures is a fraud is equally wrong.

3. No amount of inoculation or anything else will make up for an imperfectly prepared seed bed, poor soil, bad season, and poor cultivation.

4. The results obtained and the cost of preparing do not justify the enormous retail price of $2.00 per acre charged by commercial concerns last spring.

5. The Experiment Station will continue to supply farmers of the state with the cultures at twenty-five cents per acre. In turn, the farmers are requested to give the material a fair trial and report results. Those ordering material are also requested to write name and address plainly, as packages have miscarried and failed of delivery in many instances on account of improper address.